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⑤④ **Determining a hierarchy of pilliated organisms and a vaccine prepared from such organisms.**

⑤⑦ The finding has been made that immunologically related pilliated organisms exhibit a hierarchic relationship wherein the cross-reactivity of the antisera to first pili against second pili in the series is greater for the senior members of the series than the cross-reactivity of the first pili with the corresponding antisera from second pili. This asymmetric relationship leads to the situation where the juniormost pili in the series may be utilized to detect the presence of all members of the family senior to it and conversely the seniormost pili may be utilized to protect against infection by all strains junior in the series.

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DETERMINING A HIERARCHY OF PILIATED ORGANISMS
AND A VACCINE PREPARED FROM SUCH ORGANISMS

This invention relates to determining a hierarchy of piliated organisms and a vaccine prepared from such organisms and has its most important application to the preparation from a single strain of organism a vaccine effective against injection by a plurality of strains.

The presence of pili on certain organisms was noted many years ago and the first isolation of such material was reported in 1959 by Brinton in Nature 183, 782-786. Since that time many piliated organisms have been identified. Among these may be mentioned:

Neisseria gonorrhoeae
Pseudomonas aeruginosa

Proteus vulgaris
Vibrio cholerae
Bordatella pertussis
Klebsiella species
Enterobacter species
Corynebacterium renale
Flavobacterium species
Shigella flexneri

Escherichia coli
Neisseria meningitidis,
lactamica
Moraxella species
Salmonella species
Falvobacterium species
Serratia species
Corynebacterium diphtheriae
Actineobacter species
Aeromonas species

The pili of these various organisms may be separated from the cells and from cell debris and provide pilic material which, when injected into an immune-reponsive system, will cause the formation of antibodies to said pilic material.

Heretofore, the immunological relationship of members of a particular group of immunologically cross-reactive piliated organisms has been unclear. There have been reasons to suppose that the existence of a common antigenic

factor as well as the existence of individual determinants or groups of determinants specific to each particular strain.

Heretofore, the nature of this relationship has not
5 been specified.

According to the invention it has been found that a well definable hierarchial relationship can be provided between all members of the group of the immunologically cross-reactive piliated organisms, that is to say, where
10 organisms are piliated and the pili thereof may be isolated and purified and utilized to form antisera containing antibodies to said pili, the organisms may be arranged in a hierarchial order. It has been the surprising finding of the present work that within such an immunologically cross-
15 reactive group the degree of cross-reactivity between the antiserum of a first strain and the pili of a second strain in the group will not be the same as the degree of cross-reactivity between the antiserum to the pili of said second strain with the pili causing the antibodies to said first
20 strain.

The surprising difference has led to a mode of ranking members of an immunological group in a clearly defined hierarchy.

25 In carrying out this method, pili of the group of piliated organisms are prepared free of cell material and cell debris. The pili are then provided to an immune responsive system, such as, for example, mice or rabbits, and the intisera to said pili containing the atribodies
30 to said pili are then isolated in the usual manner.

5 Cross-reaction experiments of the type well
known to the art, for example, ELISA Tests, are
carried out between the pili and the antisera.
Suitably, these results are translated on a table
10 in matrix form showing the antisera on the columns
and the corresponding pili on the rows. Such a classifica-
tion will provide the homologous reaction readings
between a particular pilus and the antiserum correspond-
ing thereto upon the diagonal. The diagonal numbers
are then normalized to the same predetermined value
and the corresponding titers for all heterologous
15 pili against each antiserum adjusted accordingly.
The resultant Table will indicate the hierarchial
relationship.

20 Upon rearrangement of this normalized Table,
in the order of seniority, it will be seen that
a remarkable but consistent asymmetry exists between
the titers of the antisera with the pili. The titers
obtained between the antiserum to the pili of a
more senior strain and the pili of a more junior
strain is always greater than the titer obtained
25 between the pili of the said more senior strain
with the antiserum to the pili of the said more
junior strain. This relationship holds within families
of immunologically related pili.

30 This remarkable result has led to the conclusion
that within any such group the senior organisms
contain an immunological factor which will provide
an immune response against all organisms in the
hierarchy equal in rank or junior to it, thus providing
a vaccine inducing protection against infection

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5 therefrom. Conversely, the juniormost organisms
will contain a factor common to all organisms senior
to them in the hierarchy, thus enabling the juniormost
10 organism to be utilized for the purpose of detecting
antibodies to members of the family senior thereto.

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EXPERIMENTALEXAMPLE IMethod of Growth of Organisms - General

5 The meningococci were routinely propagated on GC agar base medium (Difco) plus 10% DSF (defined supplement with iron, a solution of 31% glucose, 0.78% L (+)-glutamine, and 0.039% ferric nitrate) and 1.5×10^{-5} % thiamine pyrophosphate (Brinton et al., 1978).

10 When the meningococci were grown for pilus production, the organisms were seeded on the supplemented GC agar medium described above omitting the thiamine pyrophosphate. About 500 ml of medium were poured into a sterile 39 cm x 26 cm x 2.3 cm aluminum tray with a sterile aluminum lid. Inoculum for the
15 tray was prepared by suspending a 20 to 24 hour growth of a desired meningococcal colony type from a petri dish in 0.7% casamino acid (Difco) and 0.02% thiamine pyrophosphate and spreading it over the GC agar surface with a long handled glass spreader at a cell density
20 of about 10^9 cells per tray. The petri dish was inoculated with 5 to 10 colonies of the desired colony types in each of the four quadrants. Both the inoculum and production plates were incubated for 20 to 24 hours at 35°C in a 80% humidity-5% carbon dioxide
25 incubator (Forma Scientific Co.).

EXAMPLE IIPilus Purification

The meningococci were grown as described above. After approximately 20 to 24 hours incubation, they were harvested and processed. The exact procedures were strain dependent and there was no general pilus pilus purification method that might apply to all meningococci. However, all the purification methods described below made use of the strong tendency of pilus rods to form longitudinal aggregates (paracrystals, Brinton, 1965) under some solvent conditions and the dissociation of the aggregates under another set of conditions.

a) N. Meningitidis ATCC 13090

The meningococci were harvested by washing the agar surface with a small volume of Tris . HCl buffered saline 0.05 M, pH 9.0. The suspended growth was then aspirated into a vacuum flask. The growth from all trays was pooled. After the suspensions were blended at 4000 rpm in small aliquots of 200 ml for 2 minutes in the 450 ml cup of the Sorvall omnimixer, the cells were removed by centrifugation at 13,000 g for 30 minutes. The supernatant was then further clarified at 23,000 g for an hour while the cell pellet from the previous centrifugation step was reextracted. The two supernatants were combined and dialyzed against two changes of 15 x volume of 0.1 M phosphate buffer at pH 6,8. The crystallization of pilus aggregates was evident by the appearance of streaming birefringence inside the dialysis sac

when it was swirled. The aggregates were removed by centrifugation at 13,000 g for an hour and the supernatant was discarded. The pellet was resuspended by magnetic stirring for about 45 minutes in a minimal volume of Tris · HCl buffered saline such that very few or no crystals were observed at 320 x magnification under a Leitz darkfield microscope. The solution was then clarified at 23,000 g for an hour. The pellet was discarded and the supernatant was dialyzed against 0.1 M phosphate buffer at pH 9.0. Usually three to four cycles of solubilization and crystallization were sufficient to achieve a reasonably pure preparation as judged by SDS-PAGE. The purified pilus crystals were stored in the aggregated state in 0.02% sodium azide.

b) N. Meningitidis NRC 1597

The harvesting procedure, the speed and duration of blending and centrifugation were the same as in the purification method described for ATCC 13090. The modifications were as follows: harvesting buffer, 0.02 M phosphate buffered saline at pH 6.8; crystallization conditions, addition of 4M stock $MgCl_2$ to 0.1 M Mg^{++} ; solubilization buffer, 0.03 M phosphate buffered saline at pH 6.8.

c) N. Meningitidis NRC 1667

The cells were grown and harvested in phosphate buffered saline at pH 6.8. The suspension was then blended at 4000 rpm for 2 minutes in 200 ml aliquots. The supernatant from a 13,000 g x 30 minute spin was further clarified by centrifuging at 23,000 g for an hour. Solid sodium dextran sulfate 2000

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(Pharmacia) was added to the solution to a final concentration of 1%. After standing for 1 hour at 4°C, 0.15 ml of 3 M stock KCl per ml of solution was added to precipitate the dextran sulfate. This cloudy suspension was then clarified by a low speed centrifugation (2000 or 4000 g for 15 minutes) and a high speed centrifugation (23,000 g for 1 hour). The resulting supernatant was adjusted to 5% concentration of PEG-6000. Crystals appeared immediately but the process of crystallization was allowed to proceed at 4°C for several hours or over night. The crystals were then collected at 13,000 g for 1 hour, redissolved in a minimal amount of solvent buffer and clarified at 23,000 g for an hour. After two such cycles, the preparation was quite pure as judged by SDS-PAGE. However, UV absorption spectroscopy showed the presence of nucleic acids. To remove nucleic acid contaminants, a preparative CsCl density gradient equilibrium centrifugation was performed. At the end of the run, fractions were collected by puncturing the bottom of the centrifuge tube. Fractions around the protein peak at density of 1.32 gm/cc were pooled, dialyzed against several changes of phosphate buffered saline, pH 6.8, and finally recrystallized at 3% PEG concentration. The purified pili were stored in 0.02% sodium azide, phosphate buffered saline at pH 6.8. This procedure is illustrated by the accompanying flow sheet.

Subsequent studies indicated preliminary fractionation by sodium dextran might be omitted if the solution were adjusted to 0.5 M sodium chloride prior to crystallization.

d) N. Meningitidis NRC 1700

The harvesting, blending and centrifugation procedures were the general steps described for preparation

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of ATCC 13090 and NRC 1597 meningococcal pili. The buffer systems were like that of the gonococcal pilus preparation method. The harvesting and solubilizing buffer was 0.63 M monoethanolamine hydrochloride at pH 10.5. The crystallization buffer contained 10% saturated ammonium sulfate and 1.7% PEG-6000.

EXAMPLE III

Density Gradient Equilibrium Centrifugation

Two kinds of CsCl density centrifugation were used in this study: the analytical and the preparative methods.

To determine the buoyant density of a pilus protein, the analytical procedure was followed. About 0.7 mg of pilus protein was dissolved in Tris-HCl buffered saline at pH 9.0; solid CsCl was added to the solution such that the average solution density was about 1.3 gm per ml. The solution was then centrifuged in a SW 41 Ti rotor at 35,000 rpm for 40 hours at 4°C. At the end of the run, the bottom of the centrifuge tubes was punctured and fractions were collected dropwise. Refractive index was determined at room temperature using a Zeiss Abbe refractometer, and the protein content of each fraction was determined either by a protein-dye binding assay or by its absorbance at 280 nm. The density of the protein peak at 4°C was estimated from a refractive index - CsCl density conversion table constructed from data available in the International Critical Tables (Vol. 3, page 94, 1928).

EXAMPLE IVPreparation Density Gradient Equilibrium

Preparative CsCl density centrifugation was used as an intermediate step in the purification of NRC 1667 meningococcal pili. Preformed gradient of six steps of CsCl was employed. Each step contained 3.0 ml of CsCl and the density ranged from 1.18 gm/cc to 1.28 gm/cc (1.18, 1.20, 1.22, 1.24, 1.26, 1.28). Up to 19 ml of a partially purified pilus solution could be accommodated per centrifuge tube. The solution was spun at 22,000 rpm for 40 hours in a SW 27 rotor at 4°C. Collection and assay of the fractions were described as in the analytical centrifugation method.

EXAMPLE VPreparation of the Antigens and the Antisera

To ensure that pure pili were used in the preparation of antisera, the purified meningococcal pili were subjected to a final purification step of CsCl density gradient centrifugation. This highly purified preparation was then injected subcutaneously in the dorsal area of the neck of a pre-bled 3 to 5 pound New Zealand white rabbit. The rabbit received approximately 300 to 500 ug of pilus protein in a 1:1 suspension of pili and Freund's incomplete adjuvant (Difco). The injection was to be repeated three times at approximately 10 day intervals. At the end of the injection schedule, the animal was bled from the ear.

Blood was drawn from the central artery of the rabbit ear without the use of any anesthetics on the animal. The hair on the back of the ear was shaved off and the artery was temporarily distended

by rubbing the skin with xylene. Blood was collected into a sterile syringe fitted with a 25 guage needle. Ten to twenty ml of blood could be obtained this way.

5 Blood was then transferred to a sterile centrifuge tube and allowed to clot at room temprature for 30 minutes. After breaking up the clot and . freeing it from the vessel wall, the entire specimen was refrigerated overnight at 4°C. The tube was
10 then spun at 4000 g for 30 minutes; and the serum removed and saved.

This antiserum prepartion was distributed in small aliquots, frozen in liquid nitrogen and stored at -20°C.

15 EXAMPLE VI

Enzyme Linked Immunosorbent Assay ("ELISA")

Description of this assay and the interpretation of the results were discussed in detail by Brinton
20 et al. (1978). The first antibody was obtained from rabbits immunized with purified pili. The second antibody was goat anti-rabbit IgG (light and heavy chains) conjugated with horseradish peroxidase (Cappel Inc.). When human sera were assayed, goat anti-human IgG conjugate was used.

25 Serum titers were calculated from the absorbance and the dilution factor, and finally standardized by comparison with serum controls. The standard curve was a plot of absorbance vs relative

serum concentration obtained by reacting a serum standard at several dilutions with its homologous antigen. Absorbances between 0.1 and 1.0 were used for calculations. Titers reported were geometric means of two runs. Inclusion of standards in every assay series eliminated titer fluctuations due to variations in the test reagents.

Calculation of Rabbit Serum Titers

The ELISA titers of rabbit sera were calculated from a standard curve which was based on the reaction of a standard rabbit serum (anti-GC 3-2) at several dilutions with its homologous antigen (GC 3-2 pili). This serum was arbitrarily set to have a titer of 10.000.

Titers of unknown sera were determined from the linear regression line of the serum standard. These titers when multiplied by the dilution factor gave the titers of undiluted sera.

EXAMPLE VII

20 Immunological Characterization of Meningococcal Pili

1. Antigenicity of Meningococcal Pili

When rabbits were hyperimmunized with the pilus antigens, elevated levels of specific antibodies were found in the antisera using the enzyme-linked immunosorbent assays (Table 1).

Table 1

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a: Homologous and heterologous titers of rabbit anti-meningococcal sera

Pili	Antisera				
	ATCC 13090	NRC 1597	NRC 1667	NRC 1700	pooled normal
ATCC 13090	3005	691	135	53	7
NRC 1597	115	2360	329	82	7
NRC 1667	784	147	838	67	5
NRC 1700	560	603	210	407	12

b: Normalized titers

Pili	Antisera			
	ATCC 13090	NRC 1597	NRC 1667	NRC 1700
ATCC 13090	100	29	17	13
NRC 1597	4	100	40	20
NRC 1667	26	6	100	17
NRC 1700	19	26	25	100

Average heterologous titer = 20.1 ± 2.9

2. Cross-reactivities among Meningococcal Pili

Table 1a shows the homologous and heterologous ELISA titers of anti-pilus sera prepared in hyperimmunized rabbits against purified meningococcal pili.

5 Considerable cross-reactivity was observed. Average cross-reactivity among the four meningococcal pili was about 20.1 ± 2.9 (Table 2b). Asymmetric cross-reactivity was observed for the meningococcal pili.

3. Cross-reactivity with Other Somatic Pili

10 Hyperimmune rabbit anti-meningococcal pilus sera and antisera directed against other kinds of somatic pili were tested for cross-reactivity. Table 2a shows the normalized homologous and heterologous ELISA titers of antisera prepared in rabbits
15 against four meningococcal and four gonococcal.

Among the three kinds of somatic pili examined here, gonococcal and meningococcal pili were found to have cross-reacting antibodies.

Average cross-reactivity of pili within
20 the species of the meningococcus and the gonococcus are $12.8 \pm 2.4\%$ and $20.1 \pm 2.9\%$, respectively. Asymmetric cross-reactivities between meningococcal and gonococcal pili were also observed (grouped data, Table 2b). The average normalized heterologous
25 titers of the gonococcal pilus antisera for meningococcal pili was about $5.5 \pm 1.1\%$; while the average heterologous titer of antimeningococcal pilus sera was about $16.9 \pm 2.3\%$.

-15-

Test results can similarly be obtained from GC pili derived from e.g. Pittsburg 4-2 (ATCC 31151), CDC 3-2 (ATCC 31150) strains and on pili derived from N. lactamica.

Table 2

a: Normalized homologous and heterologous titers of rabbit antisera against purified pili

Antisera									
		<u>N. gonorrhoeae</u>				<u>N. meningitidis</u>			
Pili		3-2	M-2	Nor-2	C-2	13090	1597	1667	1700
<u>GC</u>									
Pittsburg (ATCC 31149)	3-2	100	10	7	16	5	28	16	17
CDC (ATCC 31148)	M-2	10	100	3	11	3	35	17	24
Norfolk (ATCC 31208)	2-2	33	16	100	20	3	25	14	19
CDC (ATCC 31207)	C-2	16	9	2	100	7	26	18	14
<u>MC</u>									
ATCC	13090	8	3	2	7	100	29	17	13
NRC	1597	8	7	1	7	4	100	40	20
NRC	1667	1	1	1	5	26	6	100	17
NRC	1700	8	15	2	12	19	26	25	100

b: Average cross-reactivities (average heterologous titers) between N. gonorrhoeae, N. meningitidis and E. coli somatic pili

Pili	Antisera		
	<u>N. gonorrhoeae</u>	<u>N. meningitidis</u>	<u>E. coli</u>
<u>N. gonorrhoeae</u>	12.8 ± 2.4	16.9 ± 2.3	1.5 ± 0.3
<u>N. meningitidis</u>	5.5 ± 1.1	20.1 ± 2.9	1.5 ± 0.9

Grouped data

4. Cross-absorption Experiments

Exhaustive absorption experiments confirmed that pili of N. meningitidis and N. gonorrhoeae share common antigenic determinants. Table 3a shows ELISA titers of the pilus antisera absorbed with a heterologous meningococcal pilus and Table 3b shows the titers of the same set of antisera absorbed with a gonococcal pilus. Absorptions of the antipilus antisera with heterologous neisserial pili significantly reduced the magnitude of the heterologous titers.

5

10

Table 3

a: Normalized homologous and heterologous titers of antisera absorbed with NRC 1597 meningococcal pili

Pili	Antisera						
	<u>N. gonorrhoeae</u>				<u>N. meningitidis</u>		
	3-2	M-2	Nor-2	C-2	13090	1667	1700
GC 3-2	100	2	6	3	2	6	8
M-2	1	100	1	1	1	2	15
Nor-2	26	8	100	7	0	3	3
C-2	1	5	0	100	3	1	1
MC 13090	1	0	0	0	100	2	6
1597	0	0	0	0	0	1	3
1667	0	0	0	0	23	100	6
1700	0	9	0	0	13	6	100

b : Normalized homologous and heterologous titers of antisera absorbed with M-2 gonococcal pili

Pili	Antisera						
	<u>N. gonorrhoeae</u>			<u>N. meningitidis</u>			
	3-2	Nor-2	C-2	13090	1597	1667	1700
GC 3-2	100	6	1	1	0	2	4
M-2	0	0	0	0	8	1	6
Nor-2	19	100	3	0	1	4	3
C-2	1	0	100	3	5	6	7
MC 13090	0	0	0	100	7	4	7
1597	0	0	1	1	100	25	12
1667	0	0	1	22	0	100	14
1700	0	0	0	14	3	12	100

EXAMPLE IX

The final preparation of the pilus vaccine may be exemplified by the preparation of pilus vaccine from N. meningitidis 1597. The recrystallized pill
5 are dialyzed against phosphate-buffered saline pH 6.8, ionic strength 0.15 in which buffer the pilus crystals are solubilized. The vaccine is sterilized by filtration through 0.45 micron pure site membrane filters. The pili thus prepared are of quality
10 sufficient to pass the standards of the Bureau of Biologics, Food and Drug Administration, for general safety, sterility and potency.

The vaccine is administered parenterally by subcutaneous or intramuscular injection. Since
15 the pili are solid, any pharmaceutically acceptable suspending medium may be employed. The concentration of pili in the vehicle is not critical. The sole criterion of desirability being that the pili shall be sufficiently finely divided to provide a suspension
20 which meets generally accepted standards of syringeability. A concentration of 0.1 to 2.0, preferably about 1.0 mg of pilus protein per ml, of suspending medium is especially suitable.

It is generally preferred to administer
25 the vaccine composition in more than one dose separated by a pre-determined time factor. This time factor is selected to permit the formation of an adequate titer of antibodies to the pili in the injected subject.

30 Since there are no local or systemic toxic effects engendered by the injection of vaccine,

-19-

there appear to be no upper limits to the dosage administered. It has been found suitable, however, to administer between 1 and 1000 micrograms of pili per kilogram of body weight suitably about 60 micrograms of body weight in each injection.

Utilizing the procedures disclosed above, pili derived from N. meningitidis and N. lactamica, in particular N. meningitidis, are utilized to protect against infection of vertebrate subjects not only against homologous, i.e., N. meningitidis infection but also against N. gonorrhoeae infection.

Table 4

ASSIGNMENT OF SENIORITY

Strain	rank by pili ^a	rank by serum ^a	combined rank ^b
MC 13090	6	3	4
1597	7	8	8
1667	8	7	8
1700	3	6	4
GC 3-2	2	4	3
C-2	5	5	5
M-2	4	2	3
Nor-2	1	1	1

data from table 1 a

a, ranking method by A. Labik and C. Brinton

rank by pili

(i) determine the average heterologous titer for each pili

(ii) rank the pili based on the average titers. The most junior pili has the highest titer (rank # 1).

rank by serum

(i) determine the average heterologous titer for each serum.

(ii) rank the serum based on the average titer. The anti-serum to the most junior antigen has the lowest titer (rank # 1).

b, combined rank = geometric mean of the two ranks,

Table 5

AMINO ACID COMPOSITION OF MENINGOCOCCAL PILI

Amino Acid	ATCC 13090	NRC 1597	NRC 1667	NRC 1700
AsX	23	26	17	28
Thr	15	18	18	10
Ser	20	17	18	22
Glx	18	21	13	18
Pro	6	6	4	5
Gly	14	15	15	16
Ala	34	26	23	38
Cys(1/2)	2	2	2	3
Val	16	14	11	15
Met	2	2	4	2
Ile	8	10	8	9
Leu	10	12	9	11
Tyr	6	7	6	7
Phe	1	2	5	1
His	3	3	2	3
Lys	14	13	13	16
Arg	5	6	3	6
Trp	4	4	2	5

TABLE 6

BUFFER AND STOCK SOLUTIONS

Buffers

TBS : 0.05 M Tris(hydroxymethylaminomethane) hydrochloride buffered saline
8.5 gm NaCl (Fisher)
6.0 gm Tris (Sigma 7-9)
add water to one liter
adjust pH to 9.0 with concentrated HCl (Fisher)

PBS : 0.02 M sodium phosphate buffered saline at pH 6.8
1.4 gm sodium dihydrogen phosphate (Fisher)
1.4 gm disodium hydrogen phosphate (Fisher)
6.4 gm NaCl
add water to one liter

PB : 0.1 M sodium phosphate buffer at pH 6.8
7.1 gm sodium dihydrogen phosphate
6.9 gm disodium hydrogen phosphate
add water to one liter

EAB : 0.63 M monoethanolamine hydrochloride buffer at pH 10.5
37.5 ml monoethanolamine (Fisher)
957 ml water
6.3 ml concentrated HCl

Stocks

KCL : 3 M potassium chloride
223.7 gm KCl (Fisher)
add water to one liter

PEG : 30% (W/W) polyethylene glycol
30 gm PEG-6000 (Carbowax)
70 gm water
dissolve by stirring and filter through 0.45 um Millipore filter

MgCl₂ : 4 M magnesium chloride
381 gm MgCl₂ (Fisher)
add water to one liter
filter through 0.45 um Millipore filter

SAS : saturated ammonium sulfate at pH 10.5
760 gm ammonium sulfate (Baker)
1000 ml 0.63 M EAB at pH 10.5
adjust pH to 10.5 before use

CLAIMS:

1. A method of determining the hierarchic position of a strain of a group of strains of immunologically related piliated organisms, the antisera against the pili of said strains cross-reacting immunologically against the pili of said strains comprising the steps of:
- a) providing the pili of the organisms comprising said group, said pili being substantially free of cell material and cell debris,
 - b) providing said pili to an immun responsive system to generate antisera to said pili,
 - c) isolating said antisera,
 - d) reacting the aforesaid pili with the thus-obtained antisera and measuring the degree of cross-reactivity between said pili and said antisera,
 - e) normalizing the degree of cross-reactivity of d) to designate a common value for all homologous pilus/antiserum reaction,
 - f) arranging said strains in order of reactivity of antisera with pili in said group to provide that the titer of antisera against pili from a more senior strain reacting with a pili of a more junior strain is greater than the titer of the antisera against pili from said more junior strain reacting with pili from said more senior strain whereby the hierarchy of the group may be determined.
2. A method of determining the presence of antibodies to pili of immunologically related group of piliated organisms which comprises:

- a) determining the identity of the juniormost strain of the group in accordance with the procedures of claim 1,
- b) reacting the pili derived from said junior strain with antisera suspected of belonging to the hierarchial group whereby an immunological cross-reaction with the antisera and the pili indicates the presence of antibodies in the antisera equivalent or senior to the junior strain in the hierarchy.
- 5
- 10 3. A material derived from pili of Neisseria meningitidis which is substantially free of cell material and cell debris.
4. A material according to claim 3 which shows immunological cross-reactivity against antisera containing antibodies
- 15 againsts substantially cell-free or cell debris-free pili derived from N. gonorrhoeae.
5. A material according to claim 4 wherein said antisera are antisera containing antibodies against the pili of N. gonorrhoeae derived from at least one of the strains
- 20 designated as Pittsburg 3-2 (ATCC31149), CDC 3-2 (ATCC 31150), CDC M-2 (ATCC 31148), and Pittsburg 4-2 (ATCC 31151).
6. A material according to claim 3, or claim 4, wherein the strain of N. meningitidis is ATCC 13090, NRC 1597, NRC 1667, or NRC 1700.
- 25 7. A vaccine composition effective against infection by N. meningitidis, N. lactamica, or N. gonorrhoeae comprising a material of claim 3, claim 4 or claim 6 in a pharmaceutically acceptable medium.

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